The limitation "One or more copies" was present in previously pending claims 16-34 (many of which were allowed) in the Office Action of 12/29/05. So this amendment simply restores the limitation "One or more copies" to claims 17-34. This limitation has remained unchanged in allowed independent claim 16. Indeed, allowed independent claim 16 is unchanged and has not been amended since the last Office Action of 12/29/05.

Type (1) oligonucleotides are allele specific and are clearly supported (see paragraphs [0143], [0324], and [0346]). **Since the scope of all of claims 17-34 has been narrowed by the amendment to include only type (1) oligonucleotides, the scope of each of these claims is still within the scope of previously allowed (in the Office Action of 12/29/05) independent claim 16. Again, allowed independent claim 16 is unchanged and has not been amended since the last Office Action of 12/29/05.**

Regarding the new claims 57- 80

Regarding new claim 57, this claim format of "genotyping" is described in paragraphs [0242] and [0243]. In addition, applicants cite the cases of Ex parte Kelly (173 USPQ 743), Ex parte Britton (154 USPQ 321) and Ex parte Macy (132 USPQ 545). These cases indicate that single step method claims are patentable.

Regarding new claims 58-60 the limitations *"exact, true bi-allelic"* and *"thousands of covering markers are from one chromosome"* in these claims have been discussed in the Remarks section in the previously filed Amendment/Response of May 30, 2006; see specifically bottom p. 13 and pp. 14-15 of the 5/30/06 Amend/Response. The limitation *"thousands of covering markers"* has been discussed in the Remarks Section of previously filed amendment/responses (see especially pp. 15-18 under claims 14 and 24 of the Supplemental Amendment of Nov 20, 2005) and claims with this limitation have already been allowed by the Examiner.

Regarding new, independent claim 61 This claim is supported by [0231], [0155] and [0152]. As is known in the art, the activity of obtaining genotype data/sample allele frequency data for linkage studies is generally considered to be a separate activity from actually performing the study. Thus, for example, apparatus to obtain such data are generally regarded as separate from the studies, see e.g. [0244]-[0250] and bottom [0163]. Other support is in [0208] and [0209].

Applicants again respectfully cite the cases of Ex parte Kelly (173 USPQ 743), Ex parte Britton (154 USPQ 321) and Ex parte Macy (132 USPQ 545). These cases indicate that single step method claims are patentable. And these cases also cite the fact that *"rejection of method claims as mere function of machine claims must fail"* as in MPEP 2173.05(v).

Applicants respectfully submit that because the invention of claim 61 is closely related to that of allowed independent claim 39, a new search for patentability is not required. Applicants respectfully submit that allowed independent claim 39 (with dependent claims 40 – 42) are essentially patentable by virtue of being processes that obtain data at a novel/unobvious group of markers. Specifically this is a group of two or more covering markers that systematically cover a CL-F region New claim 61 is similarly a novel/unobvious process of obtaining data at a group of two or more covering markers that systematically cover a CL-F region. And applicants respectfully submit that the invention defined by claim 61 is similarly patentable as the inventions of allowed claims 39-42.

In addition, applicants note that allowed independent claim 39 and allowed dependent claims 40-42 are within the scope of new independent claim 61. Independent process claim 61 does not recite the phrase *"determining information on the presence or absence of each allele of each b-allelic marker .....in the chromosomal DNA of one or more individuals"* as independent process claim 39 does. Therefore it is possible for the process of claim 61 to obtain the data directly with a computer (see [0173] and [0208]) or to obtain the data using "*information on the presence or absence of each allele of each b-allelic marker .....in the chromosomal DNA"* as is done in claim 39.

Regarding new dependent claims 62-67 The limitations in claim 62 are essentially similar to those in claim 7. Claim 7 was discussed on page 13 of the Amend/Resp. of 5/30/2006; **claims with these limitations have been previously allowed.** For the limitation "genotype data" (claim 64), see "(1)" in the definition of genotype data/sample allele frequency data in [0148]; other added limitations in claims 62- 68 have been discussed above. The limitations "0.2" and "12 cM" are supported for example by [0180], [0181] and [0226].

Regarding new claim 68 the added limitations in this claim are similar to previously filed claims 8, 54 and 56. Claim 8 was discussed on p. 13 of the Amend/Resp. of 5/30/2006. The limitation "thousands of covering markers from the [or one] chromosome" is discussed on pp. 14 and 15 of the Amend/Resp. of 5/30/06.

Regarding new dependent claims 69-70, type (2) oligonucleotides with utility as PCR primers are described, see bottom half of paragraph [0143], p. 11. Other limitations in these claims were discussed above. For example, limitations in claim 69 are similar to those in claim 62 (discussed above). The limitations in claim 70 "0.2" and "12 cM" are discussed above under claims 62-67 and "thousands of covering markers" is discussed above under claims 58-60. Claims with the limitation "thousands of covering markers" have been previously allowed.

Regarding new independent claim 71 the limitation "oligonucleotides with utility as PCR primers to obtain genotype data/sample allele frequency data by generating a physico-chemical signal" is supported specifically by "(3) a signal (such as dye color) generated by the products of PCR ... as when oligonucleotides ... are used as primers for PCR reactions" see [0144]. Other support for oligonucleotides as PCR primers for use in obtaining genotype data/sample allele frequency data are specific examples of "Oligonucleotide Technology" in paragraph [0248] and [0249]. And more specific information in the examples in new paragraphs [0249.1], [0249.2], [0249.3], and [0249.4], that have been requested to be added to the Specification by amendment (July 15, 2006) and with the present amendment. Other general support for this limitation is in paragraphs [0244]-[0247], and [0251]-[0262]. The Examiner is also directed to paragraph [0144] where the term "physico-chemical signal" is broadly defined.

Such use of "Oligonucleotide Technology" (and of oligonucleotides as PCR primers) to "obtain genotype data/sample allele frequency data for each of two or more bi-allelic covering markers, wherein a CL-F region is systematically covered by the covering markers" is frequently described, see for example [0259] and [0163], especially at top p. 13.

The phrase "whereby the oligonucleotides include copies of a set of oligonucleotides, the set of oligonucleotides being complementary to the group of two or more bi-allelic covering markers" is not a true limitation, but merely states a result of the invention recited in the claim. **That is, the oligonucleotides (with the utility as PCR primers recited in the claim) must necessarily include copies of a set of oligonucleotides that is complementary to the group of two or more bi-allelic covering markers**. (See definition [0146].) Such a set of oligonucleotides is novel and unobvious as evidenced by the allowance of independent claim 16. **Similarly the applicants respectfully submit that the invention of new claim 71 is also novel/unobvious because it includes novel/unobvious copies of the set of oligonucleotides; and new claim 71 does not require a new search for patentability.** In addition, applicants respectfully submit that product claim 71 is patentable based on what the invention is, i.e. oligonucleotides that include copies of a set of oligonucleotides that is complementary to a group of two or more bi-allelic covering markers that systematically cover a CL-F region. And applicants respectfully submit that the patentability of product claim 71 is not essentially based on what the invention does or is intended to do.

The Examiner rejected former product claim 26 (under point 2 of the previous Office Action of 12/29/05) for failing to further limit the subject matter of a previous claim with an "intended use" (rather than structural) limitation. The "intended use" was "PCR primers". Applicants respectfully submit that the scope (or subject matter) of new claim 71 is clearly different than the scope of similar claim 17, because the scope of claim 71 includes type (2) oligonucleotides. The scope of claim 71 is also different than the scope of similar claim 69, as the scope of claim 71 includes type (1) oligonucleotides (some PCR primers are allele-specific and are type (1), see Abstract of the Wu reference [0347], which is enclosed). **Other limitations in claim 71 are similar to those in claim 62 (discussed above); claims with these other limitations have been allowed.**

Regarding new dependent claims 72-80 the limitations in these new dependent claims have been discussed above or in the Amend/Resp of 5/30/06. For example, the species limitations in claims 78 and 79 were discussed on p. 12 of the Amend/Resp of 5/30/06. Claims with the limitation "wherein the density of covering markers is at least thousands per chromosome" have been previously allowed. This limitation is discussed in the Supplemental Amend/Resp. of 11/20/2005 (see p. 19 under claim 30). In addition most of these limitations are present in other pending claims that are not essentially new.

Some further remarks for the record

Regarding the limitation "an essentially one-dimensional panel for a linkage study", such panels attempt to achieve one-dimensional closeness and linkage, including linkage disequilibrium, of a panel marker and the sought trait-causing polymorphism. The one-dimensional closeness is along the chromosomal location dimension. For example, the approximate even distribution of markers along the chromosomal dimension in a conventional linkage study (paragraphs [0018]-[0020] and [0035]) is to achieve such one-dimensional closeness. See also, for example, paragraph [0035] which refers to one-dimensional closeness in a one-dimensional perspective. Such one-dimensional techniques also favor markers with least common allele frequencies near 0.5, see [0042], [0026]. The linkage disequilibrium (including increased linkage disequilibrium) which essentially one-dimensional panels attempt to achieve is measured (or essentially measured) as δ/δ_{max} when $\delta \geq 0$ and δ/δ_{min} when $\delta < 0$. Such essentially one-dimensional panels are not based on using the principle of similarity of marker allele frequency and [possible] trait-causing polymorphism allele frequency to increase power, see for example [0029], [0285] and [0308].

The inventor, R. E. McGinnis, has shown that the increase in power that occurs when m is similar to p does not drop off dramatically as soon as the m/p ratio departs from unity as indicated by Muller-Myhsok and Abel (see for example [0029] and below). Some relevant text from the well-known letter of Muller-Myhsok and Abel (*Science*, Vol. 275, Issue 5304, 1328-1329, 28 February 1997) is as follows: *"The situation described by Risch and Merikangas corresponds to complete linkage disequilibrium, that is,* $\delta = \delta_{max}$ *with m = p, with P(tr–B) reducing to* $\gamma/(\gamma + 1)$*. In other cases, the number of necessary families increases dramatically as p differs from m even when* $\delta = \delta_{max}$*, and also as* δ *decreases. Thus, the power of association studies such as the TDT can be quite strong when there is a high probability that the allele studied is the causal allele as shown by Risch and Merikangas. In other cases, researchers should be aware that the power of such association studies can be greatly diminished as soon as the ratio m/p departs from unity and the linkage disequilibrium becomes weaker."*

Regarding the term "genotype data/sample allele frequency data" in the claims. Some claims such as claim 39 use the term "genotype data/sample allele frequency data". As noted in the Definitions section at [0148], there are three numbered possibilities ((1), (2), and (3)) for this data. Sample allele frequency data is determined from pooled DNA specimens or from calculation using genotype data at the marker for each individual in the sample, see [0147] and [0148]. Thus when *"genotype data/sample allele frequency data"* is present, then either sample allele frequency data determined from pooled DNA specimens or genotype data at the marker for each individual in the sample is present. And the term "genotype data/sample allele frequency data for a marker and a sample" has the same scope as the phrase "genotype data (at the marker for each individual in the sample) or sample allele frequency data (for the marker for the sample)". And "genotype data (at the marker for each individual in the sample) or sample allele frequency data (for the marker for the sample)" includes within its scope the numbered possibility (2) above, which is a combination.

Regarding claim 44, the phrase "genotype data/sample allele frequency data" was amended to "genotype data". Applicants respectfully submit that in this claim, the amended phrase does not change the scope of the claim, as it is still possible to use the specific apparatus recited in the claim to obtain sample allele frequency data, for example by using the apparatus with pooled DNA specimens.

The phrase "One or more copies of a set of oligonucleotides" was amended to "copies of a set of oligonucleotides". Applicants respectfully submit that in this claim, the amended phrase does not change the scope of the claim, as the specific apparatus recited in the claim (arrays, nylon membranes, chips, glass slides) have more than one copy of a complementary set of oligonucleotides attached to the apparatus.

Regarding claim 45 This claim and others includes the limitation *"wherein the group of two or more covering markers is not an essentially one-dimensional panel of markers for a linkage study"*. This limitation expressly excludes apparatus that are "essentially one-dimensional techniques" (i.e. apparatus that are designed to obtain data for an essentially one-dimensional linkage study) from the scope of the claim.

Some other claims with this limitation are claims 7, 17, and 69. Again the limitation in these claims expressly excludes "essentially one-dimensional techniques" from the scope of the claims. So, for example, an essentially one-dimensional study is expressly excluded from the scope of claim 7; and oligonucleotides designed to obtain genotype data for an essentially one-dimensional study are expressly excluded from the scope of claim 17.

Applicants hereby also rebut any presumption of surrender or disclaimer of any equivalent within the scope of any claim. Such a disclaimer is not necessary for patentability.

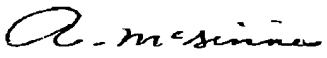
Conclusion

In this Supplemental Amendment the applicants have requested the addition of three new paragraphs to the Specification. And the applicants have also made minor amendments to previously pending dependent claims 17-34 and new claims have been added. (These amendments were made after more consideration of an issue raised by the Examiner in the previous Office Action of 12/29/05 that dealt with PCR primers.) Twenty-four new claims (including two new independent claims) have been added and one dependent claim has been cancelled. An appropriate fee for 23 extra claims including 2 independent claims has been submitted. Remarks have been made, including those indicating that the new independent claims are closely related to previously allowed claims and do not require a new search for patentability.

This Supplemental Amendment follows an Amendment/Response filed on Tuesday, 5/30/2006. The Amendment/Response of 5/30/2006 included claim amendments and responded to each point of rejection in the previous Office Action of 12/29/05 This Supplemental Amendment also follows a Supplemental Amendment of the Specification (filed on July 15, 2006). Appropriate fees are also enclosed.

For the reasons advanced above, applicants respectfully submit that the application is now in condition for allowance and that action is earnestly solicited.

Respectfully submitted,



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Enclosures, marked copies of 6 pages:

1) Saiki,et al. Proc Natl Acad Sci USA vol 86, pp.6230-6231.

2) Wu, et al., Proc Natl Acad Sci USA vol 86 pp 2757-2758.

3) Nickerson, et al., Proc Natl Acad Sci USA vol 87, pp. 8923-8924.

Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 6230–6234, August 1989
Genetics

Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes

(polymerase chain reaction/"reverse dot blots"/nonradioactive detection/*HLA-DQA* locus/β-thalassemia)

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Communicated by Hamilton O. Smith, May 9, 1989 (received for review March 2, 1989)

ABSTRACT The analysis of DNA for the presence of particular mutations or polymorphisms can be readily accomplished by differential hybridization with sequence-specific oligonucleotide probes. The *in vitro* DNA amplification technique, the polymerase chain reaction (PCR), has facilitated the use of these probes by greatly increasing the number of copies of target DNA in the sample prior to hybridization. In a conventional assay with immobilized PCR product and labeled oligonucleotide probes, each probe requires a separate hybridization. Here we describe a method by which one can simultaneously screen a sample for all known allelic variants at an amplified locus. In this format, the oligonucleotides are given homopolymer tails with terminal deoxyribonucleotidyltransferase, spotted onto a nylon membrane, and covalently bound by UV irradiation. Due to their long length, the tails are preferentially bound to the nylon, leaving the oligonucleotide probe free to hybridize. The target segment of the DNA sample to be tested is PCR-amplified with biotinylated primers and then hybridized to the membrane containing the immobilized oligonucleotides under stringent conditions. Hybridization is detected nonradioactively by binding of streptavidin-horseradish peroxidase to the biotinylated DNA, followed by a simple colorimetric reaction. This technique has been applied to *HLA-DQA* genotyping (six types) and to the detection of Mediterranean β-thalassemia mutations (nine alleles).

Differential hybridization with sequence-specific oligonucleotide probes has become a widely used technique for the detection of genetic mutations and polymorphisms (1–5). When hybridized under the appropriate conditions, these synthetic DNA probes (usually 15–20 bases in length) will anneal to their complementary target sequences in the sample DNA only if they are perfectly matched. In most cases, the destabilizing effect of a single base-pair mismatch is sufficient to prevent the formation of a stable probe–target duplex (6). With an appropriate selection of oligonucleotide probes, the relevant genetic content of a DNA sample can be completely described.

This very powerful method of DNA analysis has been greatly simplified by the *in vitro* DNA-amplification technique, the polymerase chain reaction (PCR) (7–9). The PCR can selectively increase the number of copies of a particular DNA segment in a sample by many orders of magnitude. As a result of this 10^6- to 10^8-fold amplification, more convenient assays and nonradioactive detection methods have become possible (10–12). These PCR-based assays are usually done by amplifying the target segment in the sample to be tested, fixing the amplified DNA onto a series of nylon membranes, and hybridizing each membrane with one of the labeled oligonucleotide probes under stringent hybridization conditions. However, each probe must still be individually hybridized to the amplified DNA and the process can easily become difficult in a system where many different mutations or polymorphisms occur.

One approach to address this procedural difficulty is to "reverse" the DNAs: attach the oligonucleotides to the nylon support and hybridize the amplified sample to the membrane. Thus, in a single hybridization reaction, an entire series of sequences could be analyzed simultaneously. The strategy we adopted was to immobilize the oligonucleotides onto nylon filters by ultraviolet fixation. Exposure to UV light activates thymine bases in DNA, which then covalently couple to the primary amines present in nylon (13). It seemed unlikely, however, that short oligonucleotides could be directly attached to nylon in this manner and still retain their ability to discriminate at the level of a single base-pair mismatch. Consequently, the addition of a long deoxyribothymidine homopolymer tail, poly(dT), to the 3′ end of the oligonucleotide appeared promising for several reasons. First, the poly(dT) tail would be a larger target for UV crosslinking and should preferentially react with the nylon. Second, dTTP is very readily incorporated onto the 3′ ends of oligonucleotides by terminal deoxyribonucleotidyltransferase and would permit the synthesis of very long tails (14). (Deoxyribothymidine would also be the most efficiently incorporated base if a purely synthetic route were chosen.) Third, Collins and Hunsaker (15) had shown that the presence of a poly(dA) homopolymer tail, used to introduce multiple ^{35}S labels, did not affect the function of sequence-specific oligonucleotide probes.

We have used this technique to attach oligonucleotide probes specific for the six major *HLA-DQA* DNA types (16) and the eight most common Mediterranean β-thalassemia mutations (4) to nylon filters. The target segment of the DNA sample to be tested (either *HLA-DQA* or β-globin) was amplified by PCR with biotin-labeled primers to introduce a nonradioactive tag. Hybridization of the amplified product to the immobilized oligonucleotides and binding of streptavidin-horseradish peroxidase conjugate to the biotinylated primers were performed simultaneously. Detection was accomplished by a simple colorimetric reaction involving the enzymatic oxidation of a colorless chromogen that yielded a red color wherever hybridization occurred.

MATERIALS AND METHODS

Tailing of Oligonucleotides. Oligonucleotides were synthesized on a DNA synthesizer (model 8700, Biosearch) with β-cyanoethyl *N,N*-diisopropylphosphoramidite nucleosides (American Bionetics, Hayward, CA) by using protocols provided by the manufacturer. Oligonucleotide (200 pmol) was tailed in 100 μl of 100 mM potassium cacodylate/25 mM Tris·HCl/1 mM $CoCl_2$/0.2 mM dithiothreitol, pH 7.6 (17), with 5–160 nmol deoxyribonucleoside triphosphate (dTTP or

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Abbreviation: PCR, polymerase chain reaction.

dCTP) and 60 units (50 pmol) of terminal deoxyribonucleotidyltransferase (Ratliff Biochemicals, Los Alamos, NM) for 60 min at 37°C. Reactions were stopped by addition of 100 μl of 10 mM EDTA. The lengths of the homopolymer tails were controlled by limiting dTTP or dCTP. For example, a nominal tail length of 400 dT residues was obtained by using 80 nmol of dTTP in the above reaction.

Preparation of Filters. The tailed oligonucleotides were diluted into 100 μl of TE (10 mM Tris·HCl/0.1 mM EDTA, pH 8.0) and applied to a nylon membrane (Genetrans-45; Plasco, Woburn, MA) with a spotting manifold (BioDot; BioRad). The damp filters were then placed on TE-soaked paper pads in a UV light box (Stratalinker 1800; Stratagene) and irradiated at 254 nm. Dosage was controlled by the device's internal metering unit. The irradiated membranes were washed in 200 ml of 5× SSPE (1× SSPE is 180 mM NaCl/10 mM NaH_2PO_4/1 mM EDTA, pH 7.2) with 0.5% $NaDodSO_4$ for 30 min at 55°C to remove unbound oligonucleotides. If not used immediately, the filters were rinsed in water, air-dried, and stored at room temperature until needed.

Amplification of DNA. PCR amplification of genomic sequences was performed by a slight modification of previously described procedures (9). DNA (0.1–0.5 μg) was amplified in 100 μl containing 50 mM KCl, 10 mM Tris·HCl (pH 8.4), 1.5 mM $MgCl_2$, 10 μg of gelatin, 200 μM each dATP, dCTP, dGTP, and dTTP, 0.2 μM each biotinylated amplification primer, and 2.5 units of *Thermus aquaticus* (*Taq*) DNA polymerase (Perkin–Elmer/Cetus). The cycling reaction was done in a programmable heat block (DNA Thermal Cycler; Perkin–Elmer/Cetus) set to heat at 95°C for 15 sec (denature), cool at 55°C for 15 sec (anneal), and incubate at 72°C for 30 sec (extend) by the "Step-Cycle" program. After 30 repetitions, the samples were incubated an additional 5 min at 72°C. The primers contained a single molecule of biotin attached to the 5′ end of the oligonucleotides (described below).

Hybridization and Detection of Amplified DNA. Each filter with bound oligonucleotides was placed in 4 ml of hybridization solution containing 5× SSPE, 0.5% $NaDodSO_4$, and 400 ng of streptavidin-horseradish peroxidase conjugate (SeeQuence; Eastman Kodak). PCR-amplified DNA (20 μl) was denatured by addition of an equal volume of 400 mM NaOH/10 mM EDTA and added immediately to the hybridization solution, which was then incubated at 55°C for 30 min. (During this incubation, hybridization of PCR product to immobilized oligonucleotide and binding of streptavidin-horseradish peroxidase to biotin present in the PCR product occur simultaneously.) The filters were briefly rinsed twice in 2× SSPE/0.1% $NaDodSO_4$ at room temperature, washed once in 2× SSPE/0.1% $NaDodSO_4$ at 55°C for 10 min, and then briefly rinsed twice in 2× PBS (1× PBS is 137 mM NaCl/2.7 mM KCl/8 mM Na_2HPO_4/1.5 mM KH_2PO_4, pH 7.4) at room temperature. Color development was performed by incubating the filters in 25–50 ml of red leuco dye (Eastman Kodak) at room temperature for 5–10 min. Photographs were taken for permanent records.

Synthesis of Biotinylated Oligonucleotide Primers. Primary amino groups were introduced at the 5′ termini of the primers by a variation of published procedures (18, 19). In brief, tetraethylene glycol was converted to the monophthalimido derivative by reaction with phthalimide in the presence of triphenylphosphine and diisopropyl azodicarboxylate (20). The monophthalimide was converted to the corresponding β-cyanoethyl diisopropylamino phosphoramidite by standard protocols (21). The resulting phthalimido amidite was added to the 5′ ends of the oligonucleotides during the final cycle of automated DNA synthesis by using standard coupling conditions. During normal deprotection of the DNA (concentrated aqueous ammonia for 5 hr at 55°C), the phthalimido group was converted to a primary amine, which was subsequently acylated with an appropriate biotin active ester. NHS-LC-biotin (Pierce) was selected for its water solubility and lack of steric hindrance. The biotinylation was performed on crude, deprotected oligonucleotide, and the mixture was purified by a combination of gel filtration and reversed-phase HPLC. Additional details of this procedure will be published elsewhere (22).

RESULTS

Binding and Hybridization Efficiency of Tailed Oligonucleotides. The relative efficiencies with which synthetic oligonucleotides with homopolymer tails of various lengths were covalently bound to the nylon filter were measured as a function of UV exposure (Fig. 1 *Left*). Oligonucleotides with longer poly(dT) tails were more readily fixed to the membrane, and all attained their maximum values by 240 mJ/cm² of irradiation at 254 nm. In contrast, the $(dC)_{400}$-tailed oligonucleotide required more irradiation to crosslink to the nylon and was not comparable to the equivalent $(dT)_{400}$ construct even after 600 mJ/cm² exposure. This difference is consistent with the findings of Church and Gilbert (13) that suggested light-activated thymine bases bind more effectively to nylon than do cytosine bases. The untailed oligonucleotide was also retained by the membrane in a manner that roughly paralleled the poly(dC) product.

Efficient binding of oligonucleotides to the membrane, however, does not necessarily correlate with hybridization efficiency, and so hybridization efficiency as a function of UV dosage was determined in a separate experiment (Fig. 1 *Right*). These results show a distinct optimum of exposure that changes with the length of the poly(dT) tail and is more sharply pronounced for the longer tails. Additional experiments have shown the optimal dosages to be about 20 mJ/cm² for the $(dT)_{800}$ and 40 mJ/cm² for the $(dT)_{400}$ oligonucleotides (R.K.S., unpublished observations). The peak efficiencies of the $(dT)_{400}$ and $(dT)_{800}$ constructs are around 1% (45–50 fmol of radiolabeled probe annealed to ≈3.5 pmol of tailed oligonucleotide), which is similar to the value reported by Gamper *et al.* (23) for an oligonucleotide probe hybridized to nylon-bound plasmid DNA.

Comparison of the data in Fig. 1 *Left* and *Right* for 60 mJ/cm² irradiation indicates that oligonucleotides with longer tails hybridize more effectively than can be accounted for by the additional amounts bound to the filter. This suggests a spacer effect wherein the poly(dT) tails improve hybridization efficiency by increasing the distance between the nylon membrane and the terminal oligonucleotide probe. Besides possible UV damage to the DNA itself, additional exposure causes more of the tail to become attached to the membrane, thus reducing the average spacer length and decreasing hybridization efficiency. The markedly different hybridization profile of the poly(dC) oligonucleotide is compatible with this interpretation. Because cytosines react less efficiently with the filter, hybridization efficiency reaches a plateau where loss due to UV damage and tail shortening are compensated by the fixing of new molecules (see Fig. 1 *Left*). This characteristic of cytosine may make a poly(dC) tail desirable when UV irradiation cannot be carefully controlled. Under the stringent hybridization conditions used in this experiment, no signal was detected for the untailed oligonucleotide.

DNA Typing at the *HLA-DQA* Locus. The *HLA-DQA* test is derived from a PCR-based oligonucleotide typing system that partitions the polymorphic variants at the *DQA* locus into four major DNA types, *DQA1* to *DQA4*, and three *DQA1* subtypes, *DQA1.1* to *DQA1.3* (16). Four oligonucleotides specific for the major *DQA* types, four oligonucleotides that characterize the *DQA1* subtypes, and one control oligonucleotide that hybridizes to all allelic *DQA* sequences (Table

Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 2757–2760, April 1989
Genetics

Allele-specific enzymatic amplification of β-globin genomic DNA for diagnosis of sickle cell anemia

(genetic diseases/base-pair mismatch/DNA polymerase/oligodeoxyribonucleotide/polymerase chain reaction)

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Communicated by Eugene Roberts, December 27, 1988 (received for review December 12, 1988)

ABSTRACT A rapid nonradioactive approach to the diagnosis of sickle cell anemia is described based on an allele-specific polymerase chain reaction (ASPCR). This method allows direct detection of the normal or the sickle cell β-globin allele in genomic DNA without additional steps of probe hybridization, ligation, or restriction enzyme cleavage. Two allele-specific oligonucleotide primers, one specific for the sickle cell allele and one specific for the normal allele, together with another primer complementary to both alleles were used in the polymerase chain reaction with genomic DNA templates. The allele-specific primers differed from each other in their terminal 3′ nucleotide. Under the proper annealing temperature and polymerase chain reaction conditions, these primers only directed amplification on their complementary allele. In a single blind study of DNA samples from 12 individuals, this method correctly and unambiguously allowed for the determination of the genotypes with no false negatives or positives. If ASPCR is able to discriminate all allelic variation (both transition and transversion mutations), this method has the potential to be a powerful approach for genetic disease diagnosis, carrier screening, HLA typing, human gene mapping, forensics, and paternity testing.

Sickle cell anemia is the prototype of a genetic disease caused by a single base-pair mutation, an A → T transversion in the sequence encoding codon 6 of the human β-globin gene. In homozygous sickle cell anemia, the substitution of a single amino acid (Glu → Val) in the β-globin subunit of hemoglobin results in a reduced solubility of the deoxyhemoglobin molecule and erythrocytes assume irregular shapes. The sickled erythrocytes become trapped in the microcirculation and cause damage to multiple organs.

Kan and Dozy (1) were the first to describe the diagnosis of sickle cell anemia in the DNA of affected individuals based on the linkage of the sickle cell allele to an *Hpa* I restriction fragment length polymorphism. Later, it was shown that the mutation itself affected the cleavage site of both *Dde* I and *Mst* II and could be detected directly by restriction enzyme cleavage (2, 3). Conner *et al.* (4) described a more general approach to the direct detection of single nucleotide variation by the use of allele-specific oligonucleotide hybridization. In this method, a short synthetic oligonucleotide probe specific for one allele only hybridizes to that allele and not to others under appropriate conditions.

All of the above approaches are technically challenging, require a reasonably large amount of DNA, and are not very rapid. The polymerase chain reaction (PCR) developed by Saiki *et al.* (5) provided a method to rapidly amplify small amounts of a particular target DNA. The amplified DNA could then be readily analyzed for the presence of DNA sequence variation (e.g., the sickle cell mutation) by allele-specific oligonucleotide hybridization (6), restriction enzyme cleavage (5, 7), ligation of oligonucleotide pairs (8, 9), or ligation amplification (10). PCR increased the speed of analysis and reduced the amount of DNA required for it but did not change the method of analysis of DNA sequence variation. In this paper, we investigated whether PCR could be done in an allele-specific manner such that the presence or absence of an amplified fragment provides direct determination of genotype.

PCR utilizes two oligonucleotide primers that hybridize to opposing strands of DNA at positions spanning a sequence of interest. A DNA polymerase [either the Klenow fragment of *Escherichia coli* DNA polymerase I (5) or *Thermus aquaticus* DNA polymerase (11)] is used for sequential rounds of template-dependent synthesis of the DNA sequence. Prior to the initiation of each new round, the DNA is denatured and fresh enzyme is added in the case of the *E. coli* enzyme. In this manner, exponential amplification of the target sequences is achieved. We reasoned that if the 3′ nucleotide of one of the primers formed a mismatched base pair with the template due to the existence of single nucleotide variation, amplification would take place with reduced efficiency. Specific primers would then direct amplification only from their homologous allele. After multiple rounds of amplification, the formation of an amplified fragment would indicate the presence of the allele in the initial DNA.

MATERIALS AND METHODS

Oligonucleotide Synthesis. Oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer by the phosphoramidite method. They were purified by electrophoresis on a urea/polyacrylamide gel followed by high-performance liquid chromatography as described (12).

Source and Isolation of Human DNA. All genomic DNA samples with the exception of the β-thalassemia DNA were isolated from the peripheral blood of appropriate individuals. The β-globin genotype of these individuals was previously determined by hybridization with allele-specific oligonucleotide probes (4) as well as by hemoglobin electrophoresis. Thalassemia major DNA was obtained from an Epstein–Barr virus-transformed lymphocyte cell line obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository (Camden, NJ). Thalassemia DNA was isolated from the cultured cells. All DNA preparations were performed according to a modified Triton X-100 procedure followed by proteinase K and RNase A treatment (13). The average yield of genomic DNA was ≈25 μg per ml of blood.

PCR. Hβ14A (5′-CACCTGACTCCTGA) and BGP2 (5′-AATAGACCAATAGGCAGAG) at a concentration of 0.12 μM were used as the primer set for the amplification of the

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Abbreviations: PCR, polymerase chain reaction; ASPCR, allele-specific PCR.

normal β-globin gene (*a* primer set). Similarly, 0.12 μM HβI4S (5'-CACCTGACTCCTGT) and 0.12 μM BGP2 were used as the primer set for the amplification of the sickle cell gene (*s* primer set). Both primer sets directed the amplification of a 203-base-pair (bp) β-globin allele-specific fragment. As an internal positive control, all reaction mixtures contained an additional primer set for the human growth hormone gene comprised of 0.2 μM GHPCR1 (5'-TTCCCAACCATTCCCTTA) and 0.2 μM GHPCR2 (5'-GGATTTCTGTTGTGTTTC) (*hGH* primer set). GHPCR1 and GHPCR2 direct the amplification of a 422-bp fragment of the human growth hormone gene. All reactions were performed in a vol of 50 μl containing 50 mM KCl, 10 mM Tris·HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, template DNA (0.5 μg/ml), and 0.1 mM each dATP, dCTP, dGTP, and TTP. Reactions were carried out for 25 cycles at an annealing temperature of 55°C for 2 min, a polymerization temperature of 72°C for 3 min, and a heat-denaturation temperature of 94°C for 1 min on a Perkin-Elmer Cetus DNA thermal cycler. At the end of the 25 rounds, the samples were held at 4°C in the thermal cycler until removed for analysis.

Analysis of the PCR Products. An aliquot (15 μl) from each of the completed PCR reactions was mixed with 5 μl of 5× Ficoll loading buffer (1× = 10 mM Tris·HCl, pH 7.5/1 mM EDTA/0.05% bromophenol blue/0.05% xylene cyanol/3% Ficoll) and subjected to electrophoresis in a 1.5% agarose gel. Electrophoresis was performed in 89 mM Tris·HCl/89 mM borate/2 mM EDTA buffer for 2 hr at 120 V. At the completion of electrophoresis, the gel was stained in ethidium bromide (1.0 μg/ml) for 15 min, destained in water for 10 min, and photographed by ultraviolet trans-illumination.

RESULTS

Experimental Design. The scheme describing allele-specific PCR (ASPCR) is shown in Fig. 1. Primer P1 is designed such that it is complementary to allele 1 but the 3'-terminal nucleotide forms a single base-pair mismatch with the DNA sequence of allele 2 (Fig. 1*B*, *). Under appropriate annealing temperature and PCR conditions, there is normal amplification of the P1–P3 fragment with DNA templates containing allele 1 (homo- or heterozygous), while there is little or no amplification from DNA templates containing allele 2. In a similar way, a primer (P2) could be designed that would allow the specific PCR amplification of allele 2 but not allele 1 DNA.

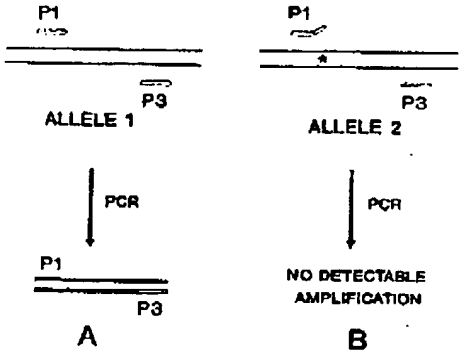

FIG. 1. Schematic representation of the ASPCR. P1 and P3, synthetic oligonucleotide primers that anneal to opposing strands of a single copy gene. P1 anneals to the region of a gene in the region of a DNA sequence variation such that its terminal 3' nucleotide base pairs with the polymorphic nucleotide of the template. P1 is completely complementary to allele 1 (*A*) but forms a single base-pair mismatch with allele 2 at the 3'-terminal position due to one or more nucleotide differences relative to allele 1 (*B*).

We designed two 14-nucleotide-long allele-specific primers, HβI4S and HβI4A, complementary to the 5' end of the sickle cell and normal β-globin genes, respectively. The oligonucleotide primers differ from each other by a single nucleotide at the 3' end, HβI4S having a 3' T and HβI4A having a 3' A corresponding to the base pair affected by the sickle cell mutation. The oligonucleotide primer BGP2 (7) complementary to the opposite strand 3' of the allele-specific primers was used as the second primer for PCR. The amplification product with these primer pairs was 203 bp. Also included in each reaction was a second pair of primers that directed the amplification of a 422-bp fragment of the human growth hormone gene. These primers were included as an internal positive control.

Discrimination Between the Normal and Sickle Cell Alleles. Genomic DNA was isolated from peripheral blood leukocytes of individuals of known β-globin genotypes (β^A/β^A, β^A/β^S, β^S/β^S). In addition, DNA was isolated from an Epstein–Barr virus-transformed cell line containing a homozygous deletion of the β-globin gene (β^{th}/β^{th}). DNA was subjected to 25 rounds of PCR using either the sickle cell-specific primer set (HβI4S and BGP2) or the normal gene-specific primer set (HβI4A and BGP2) using an annealing temperature of 55°C. The results are shown in Fig. 2*A*. It can be seen that a 203-bp fragment is observed using the sickle cell-specific primer set only with the β^A/β^S and β^S/β^S genomic DNA templates and not with the β^A/β^A genomic DNA templates. Conversely, the normal gene-specific primer set only gave rise to an amplification product with β^A/β^S and β^A/β^A genomic DNA templates. As expected, the thalassemia DNA did not give rise to a β-globin gene amplification product with either primer set. The internal growth hormone gene control gave rise to a 422-bp fragment in all samples, demonstrating that in no case was the absence of a globin-specific band due to a failure of the PCR.

In a single blind study, the DNA from 12 individuals with different β-globin genotypes was analyzed with the two primer sets. The results are shown in Fig. 2*B*. Individuals 1, 2, 3, and 5 are predicted to be β^A/β^A; individuals 6, 9, 10, and 11 are predicted to be β^S/β^S; and individuals 4, 7, 8, and 12 are predicted to be β^A/β^S. In each case, the genotype was correctly and unambiguously predicted from the pattern of fragment amplification (see legend to Fig. 2 for clinically diagnosed genotype).

DISCUSSION

The results presented above indicate the potential usefulness of ASPCR for sickle cell diagnosis. The method is rapid and the result is obtained without the use of radioactivity, since all that is required is to visualize the band on a gel with ethidium bromide staining. It should be possible to further improve the technique by elimination of the gel separation step. One strategy for this is shown in Fig. 3. As proposed recently by Yamane *et al.* (15), the two primers for the PCR could be labeled differently, one with biotin and one with a fluorescent group such as fluorescein or tetramethyl rhodamine. The product of the PCR could be captured on streptavidin-agarose and the presence of the amplified sequence could be detected with the fluorescence. In this case, if one allele-specific primer were labeled with one fluorescent group and the other were labeled with a different one, then the ASPCR could be done simultaneously.

In this study, we have used PCR primers that form either an A·A or a T·T mismatch. It is not clear that other mismatches will give equally effective discrimination. Since G·T mismatches are more stable than other mismatches (16), G·T should probably be avoided when designing primers.

Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 8923–8927, November 1990
Genetics

Automated DNA diagnostics using an ELISA-based oligonucleotide ligation assay

(DNA amplification/gene detection/genome mapping)

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Contributed by Leroy Hood, August 16, 1990

ABSTRACT **DNA diagnostics, the detection of specific DNA sequences, will play an increasingly important role in medicine as the molecular basis of human disease is defined. Here, we demonstrate an automated, nonisotopic strategy for DNA diagnostics using amplification of target DNA segments by the polymerase chain reaction (PCR) and the discrimination of allelic sequence variants by a colorimetric oligonucleotide ligation assay (OLA). We have applied the automated PCR/OLA procedure to diagnosis of common genetic diseases, such as sickle cell anemia and cystic fibrosis (ΔF508 mutation), and to genetic linkage mapping of gene segments in the human T-cell receptor β-chain locus. The automated PCR/OLA strategy provides a rapid system for diagnosis of genetic, malignant, and infectious diseases as well as a powerful approach to genetic linkage mapping of chromosomes and forensic DNA typing.**

The study of DNA sequence variants in humans is playing an important role in diagnosis of genetic and malignant diseases (1, 2). The analysis of DNA polymorphisms also serves as the fundamental tool in attempts to construct genetic linkage maps (3, 4) and in forensic analyses (5, 6). Since the majority of DNA sequence variants and polymorphisms are single nucleotide substitutions (1, 2), diagnostic techniques must accurately discriminate single base changes.

Single base variations in DNA sequences can be detected by a variety of techniques including Southern blot analysis (7) for restriction fragment length polymorphisms, allele-specific oligonucleotide hybridization (8), denaturing gradient gel electrophoresis (9), chemical cleavage of mismatched heteroduplexes (10), conformational changes in single strands (11), and allele-specific priming of the polymerase chain reaction (PCR) (12–14). These techniques have several disadvantages for automating DNA diagnosis, which include the use of radioactivity, the requirement for various hybridization conditions, and the need for electrophoresis or centrifugation.

The analysis of DNA sequence variants has been greatly facilitated by the development of rapid methods to exponentially amplify specific DNA or RNA targets. Diagnostic targets can be amplified by PCR (15–17) or by other available methods (18–21). Amplification generates specific targets with high signal/noise ratios and permits the use of less sensitive nonisotopic reporters in DNA analysis.

An alternative strategy for DNA diagnosis, the oligonucleotide ligation assay (OLA), employs two adjacent oligonucleotides (20-mers), a 5′ biotinylated probe (with its 3′ end at the nucleotide to be assayed) and a 3′ reporter probe (22–24). The two oligonucleotides are hybridized to target DNA and, if there is perfect complementarity, the enzyme DNA ligase covalently joins the 5′ biotinylated probe and the 3′ reporter probe. If the probes and target are mismatched at their junction, a covalent bond is not formed. Capture of the 5′ biotinylated probe on immobilized streptavidin and analysis for covalently linked 3′ reporters determine the nature of the probe–target interaction (matched or mismatched). The ligase assay uses a standard set of conditions to distinguish all nucleotide mismatches, and product analysis does not require electrophoresis or centrifugation (22). In this report, we describe a strategy for automating DNA diagnosis that combines target amplification by PCR with a nonisotopic analysis of DNA sequence variants by OLA.

MATERIALS AND METHODS

Robotic Workstation. A Biomek 1000 workstation (Beckman) equipped with multipipet tools and a multibulk tool was used to perform all pipetting, aspirating, and washing procedures. The workstation has been modified with a solenoid to switch wash solutions during the ELISA. All reagents for sample processing were stored in sterile 96-minitube cassettes.

DNA Samples. DNA from humans with α_1-antitrypsin, β-globin, and cystic fibrosis variants was obtained from F. Heijtmancik (Baylor University), from K. Tanaka (Harbor Hospital) and J. Korenberg (Cedar–Sinai Hospital), and from A. Osher and E. Hsu (Children's Hospital), respectively, and prepared as described (22). DNA for amplification of human T-cell receptor β-chain (TCRβ) gene segments was obtained by gently scraping cells from the lining of the buccal cavity with a sterile toothpick. Buccal cells were dislodged into a minitube containing 10 μl of sterile H_2O, covered with 75 μl of mineral oil, and placed into a 96-minitube cassette for handling by the robotic workstation. Cells were lysed with 20 μl of 0.1 M KOH and 0.1% Triton X-100 at 65°C for 20 min and neutralized with 20 μl of 0.1 M HCl and 0.1% Triton X-100.

Oligonucleotides. Amplification primers and ligation probes were assembled by using standard phosphoramidite chemistry on an Applied Biosystems 380A DNA synthesizer. Ligation probes were modified with a 5′ biotin group as described (15) or chemically phosphorylated with 5′ Phosphate-ON (Clontech) according to the manufacturer's directions. Modified probes were purified by reverse-phase high-performance liquid chromatography. Phosphorylated oligonucleotide probes (500 pmol) were labeled with dUTP-digoxigenin by mixing 100 mM potassium cacodylate, 2 mM $CoCl_2$, 200 μM dithio-

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Abbreviations: PCR, polymerase chain reaction; OLA, oligonucleotide ligation assay; TCRβ, T-cell receptor β chain; CFTR, cystic fibrosis transmembrane conductance regulator; V, variable; D, diversity; J, joining; C, constant; STS, sequence-tagged site.

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threitol, 2.5 μl of dUTP-digoxigenin (Boehringer Mannheim), and 2 μl of adenosine triphosphate (40 μM) with 70 units of terminal deoxynucleotidyltransferase (Collaborative Research) for 1 hr at 37°C. Free dUTP-digoxigenin was removed by two successive ethanol precipitations.

DNA Amplification. The robotic workstation was programmed to assemble PCR reagents [5 μl containing 20 mM Tris·HCl (pH 8.3), 100 mM KCl, 3 mM $MgCl_2$, 20 ng of bovine serum albumin per ml, the four deoxynucleotide triphosphates each at 400 μM, 0.5 μM amplification primers, 0.1% Triton X-100, and 0.05 unit of *Thermus aquaticus* DNA polymerase per well], genomic DNA (5 μl at 2 ng/μl in sterile distilled H_2O containing 0.1% Triton X-100), and 70 μl of light mineral oil in a flexible U-bottomed 96-well microtiter plate (Falcon). Genomic DNA samples were denatured at 93°C for 4 min and amplified by 40 cycles of 93°C for 30 sec, 55°C [cystic fibrosis transmembrane conductance regulator (CFTR) and TCRα constant (C_α) gene segments] or 61°C (β-globin and α_1-antitrypsin gene segments) for 45 sec, and 72°C for 90 sec in a microtiter plate thermal cycler (MJ Research, Watertown, MA). For amplification of TCRβ gene segments, 15 μl of PCR reagents (as described above) containing all six amplification primers, 15 μl of the lysed buccal samples, and 70 μl of mineral oil were added to a flexible microtiter plate. Targets were denatured at 93°C for 4 min and amplified by 20 cycles of 30 sec at 93°C, 45 sec at 61°C, and 90 sec at 72°C. Five microliters from these reaction mixtures was used to initiate a second round of amplification for each of the individual TCRβ gene segments (40 cycles; 30 sec at 93°C, 45 sec at 61°C, and 90 sec at 72°C).

Ligation Assays. Ligation reaction mixtures were assembled by the robotic workstation. Forty-five microliters of 0.25 M NaOH containing 0.1% Triton X-100 was added to amplified DNA samples. Ligation probes (200 fmol each) in 10 μl of 2× ligase buffer (100 mM Tris·HCl, pH 7.5/20 mM $MgCl_2$/2 mM spermidine/2 mM adenosine triphosphate/10 mM dithiothreitol) and 50% formamide were added to a U-bottomed 96-well microtiter plate. DNA samples were neutralized with 45 μl of 0.25 M HCl and six 10-μl aliquots were added to the microtiter plate containing the ligation probes. Samples were covered with 70 μl of mineral oil, denatured at 93°C for 2 min, cooled, and returned to the workstation for the addition of 5 μl of T4 DNA ligase (5 units/ml) (Amersham) in 1× ligase buffer. Ligations were done at room temperature (RT) for 15 min. Reactions were stopped by adding 10 μl of 0.25 M NaOH per well and, after 2 min at RT, 4 μl of 3 M sodium acetate (pH 6.5) per well. Samples were transferred to a 96-well flat-bottomed microtiter plate (Falcon) coated with streptavidin [60 μl of streptavidin (100 μg/ml) or avidin (100 μg/ml) (Vector Laboratories) for 1 hr at 37°C] and blocked 20 min (RT) before use with 200 μl of 100 mM Tris·HCl, pH 7.5/150 mM NaCl/0.05% Tween 20 (buffer A) per well with 0.5% dry milk and 100 μg of salmon sperm DNA per ml. Biotinylated probes were captured at RT for 30 min, and the plate was washed twice with 0.01 M NaOH and 0.05% Tween 20 and once with buffer A. Thirty microliters of anti-digoxigenin antibodies (diluted 1:1000; Boehringer Mannheim) in buffer A with 0.5% dry milk was added to each microtiter well. Plates were incubated 30 min (RT) and washed six times with buffer A. Substrate (30 μl of BRL ELISA amplification system per well) was added, the plates were incubated 15 min (RT), and 30 μl of amplifier was added. Spectrophotometric absorbances were taken at 490 nm by a Bio-Tek (Burlington, VT) plate reader and absorbances were directly entered into an IBM-XT computer.

Linkage Analysis. Observed haplotype frequencies were calculated for genetic linkage analysis of TCRβ gene segments with a myriad haplotype program (25). The probability of linkage disequilibrium was calculated based on the χ^2 distribution of the Q statistic described by Hedrick *et al.* (26).

RESULTS

The Automated PCR/OLA Strategy. Our strategy for automated gene analysis is shown in Fig. 1. A Biomek 1000 robotic workstation was used to (*i*) prepare targets and assemble reagents for DNA amplification, (*ii*) mix and ligate 5′ biotinylated probes and 3′ digoxigenin-labeled reporter probes on amplified DNA targets using T4 DNA ligase, (*iii*) capture 5′ biotinylated probes on streptavidin-coated microtiter plates, (*iv*) wash plates, and (*v*) detect the digoxigenin reporter coupled to biotin-labeled probes by an ELISA. Altogether, processing time for 96 samples from entry to computer read-out takes <7 hr. Overnight amplification permits processing of ligation assays from 192 DNA samples in a single day (1200 reactions, triplicates for two alleles).

Amplification Primers and Ligation Probes. A panel of amplification primers and ligation probes for known sequence variants in human DNA have been synthesized (Table 1). Two sets of probes detect mutations that cause common genetic diseases in homozygous individuals, sickle cell anemia and CF (27, 28). Another set detects a common mutation in the α_1-antitrypsin gene that, in homozygous individuals, leads to a predisposition for cirrhosis of the liver in childhood and emphysema in adults (29). The remaining probes detect

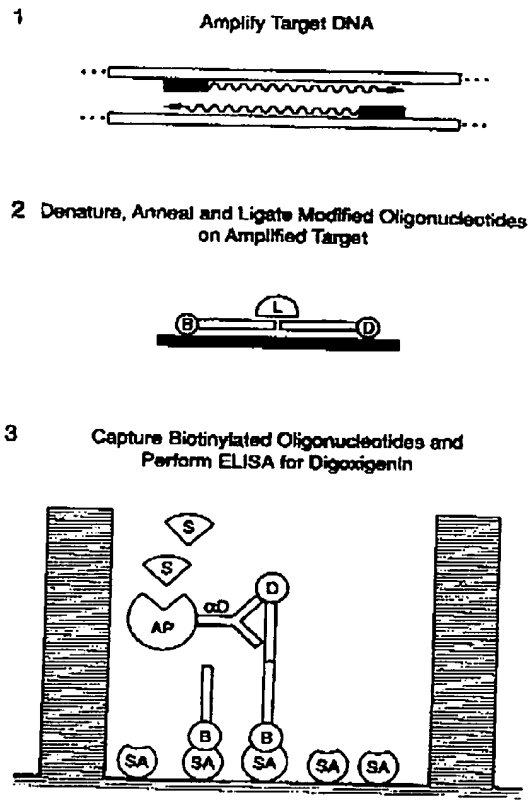

FIG. 1. Schematic diagram of the steps in the automated PCR/OLA procedure performed with a robotic workstation. The assay contains three steps: 1, DNA target amplification; 2, analysis of target nucleotide sequences with biotin (B)-labeled and digoxigenin (D)-labeled oligonucleotide probes and T4 DNA ligase (L); 3, capture of the biotin (B)-labeled probes on streptavidin (SA)-coated microtiter wells and analysis for covalently linked digoxigenin (D) by using an ELISA procedure with alkaline phosphatase (AP)-conjugated anti-digoxigenin (αD) antibodies and a substrate (S).

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